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Review

Fatty acid metabolism in *Saccharomyces cerevisiae*

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Abstract. Peroxisomes are essential subcellular organelles involved in a variety of metabolic processes. Their importance is underlined by the identification of a large group of inherited diseases in humans in which one or more of the peroxisomal functions are impaired. The yeast *Saccharomyces cerevisiae* has been used as a model organism to study the functions of peroxisomes. Efficient oxidation of fatty acids does not only require the participation of peroxisomal enzymes but also the active involvement of other gene products. One group of important gene products in this respect includes peroxisomal membrane proteins involved in metabolite transport. This overview discusses the various aspects of fatty acid β-oxidation in *S. cerevisiae*. Addressed are the various enzymes and their particular functions as well as the various transport mechanisms to take up fatty acids into peroxisomes or to export the β-oxidation products out of the peroxisome to mitochondria for full oxidation to CO_2 and H_2O.

Key words. Peroxisomes; β-oxidation; proliferation; ATP transport; carrier; yeast.

Introduction

Fatty acid β-oxidation in *Saccharomyces cerevisiae*

Peroxisomal disorders are relative newcomers in the arena of inherited diseases in humans. In recent years many different peroxisomal disorders have been identified, and great progress has been made with respect to the underlying enzymatic and genetic basis [1, 2]. On the other hand, numerous patients have been described in literature in whom the underlying defect is still unclear but appears to affect the peroxisomal β-oxidation system as concluded from the accumulation of very long chain fatty acids (VLCFAs), pristanic acid and/or di- and trihydroxycholestanoic acid in different combinations. The identification of *D*-bifunctional enzyme deficiency [3–6] and 2-methylacyl coenzyme A (CoA) racemase deficiency [7] are two recent examples of our endeavor to resolve the underlying defect in all patients with a defect in peroxisomal β-oxidation of unknown etiology [1, 8].

In order to shed more light on the functional organization and basic principles of peroxisomal β-oxidation, we and others have used the yeast *Saccharomyces cerevisiae* as a model organism. *S. cerevisiae* is able to degrade both saturated and unsaturated fatty acids [9]. In contrast to higher eukaryotes that also have mitochondrial participation, fatty acid β-oxidation in yeast is restricted to peroxisomes [10], which harbor the full enzymatic machinery to degrade fatty acids. Efficient oxidation of saturated and unsaturated fatty acids in peroxisomes does not require only the participation of a series of enzymes but also the active involvement of other gene products. Indeed, peroxisomes must be equipped with mechanisms (i) to reoxidize the NADH produced during fatty acid β-oxidation, (ii) to reduce the $NADP^+$ produced in the

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2,4-dienoyl-CoA reductase reaction, (iii) to take up fatty acids from the cytosol and (iv) to export acetyl-CoA units out of the peroxisome to mitochondria for full oxidation to CO_2 and H_2O (fig. 1).

Substrates for the β-oxidation system in *S. cerevisiae*

S. cerevisiae can use a range of different saturated long-chain fatty acids, including myristic acid (C14:0) and palmitic acid (C16:0), as sole carbon source for growth. Saturated medium-chain fatty acids, like lauric acid (C12:0) or octanoic acid (C8:0), are also substrates for β-oxidation but appear lethal when used as sole carbon source.

In addition to saturated fatty acids, *S. cerevisiae* can also utilize unsaturated fatty acids, including oleic acid (C18:1,cis-9), petroselinic acid (C18:1,cis-6), linoleic acid (C18:2,cis-9,12), linolenic acid (C18:3,cis-9,12, 15), arachidonic acid (C20:4,cis-5,8,11,14), eicosapentaenoic acid (C20:5,cis-5,8,11,14,17) and docosahexaenoic acid (C22:6,cis-4,7,10,13,16,19) as sole carbon source [C. W. T. van Roermund et al., unpublished].

Although VLCFAs, like C24:0 and C26:0, can be oxidized in *S. cerevisiae*, these fatty acids cannot be used as sole carbon source probably due to their low rate of oxidation (<1% as compared with oleate; [C. W T. van Roermund et al., unpublished]. 2-Methyl branched-chain fatty acids such as pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) cannot be β-oxidized in *S. cerevisiae* [C. W. T. van Roermund et al., unpublished].

Enzymology of the peroxisomal β-oxidation system

The actual β-oxidation of fatty acids in *S. cerevisiae* proceeds via the same set of enzymatic reactions as in mitochondria and peroxisomes from higher eukaryotes (fig. 1). This implies that a fatty acid is first activated to a CoA ester and then undergoes dehydrogenation, hydratation, again dehydrogenation and finally thiolytical cleavage.

The first step of the peroxisomal β-oxidation spiral

The first step in the β-oxidation of activated fatty acids involves the introduction of a double bond between the α and β carbon atoms. In *S. cerevisiae* only a single acyl-CoA oxidase (ScFox1p or ScPox1p) (table 1) [11] has

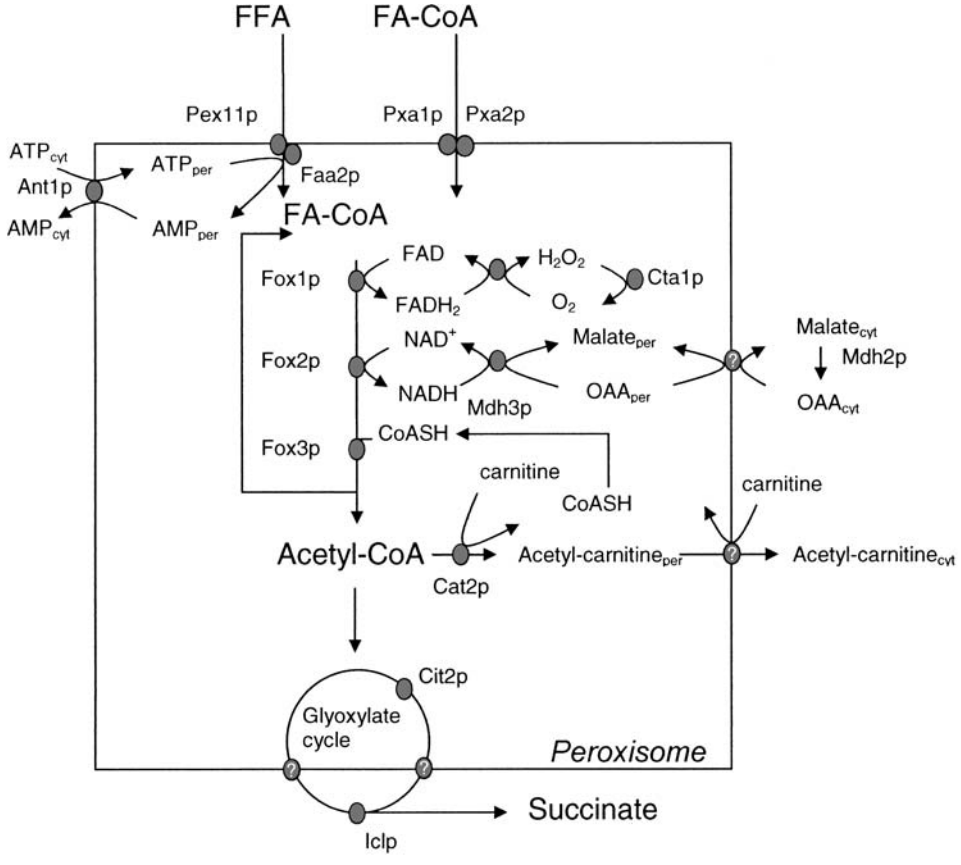

Figure 1. Metabolite transport across the peroxisomal membrane during fatty acid oxidation in *S. cerevisiae* (see table 1 for abbreviations).

Table 1. Genes encoding proteins involved in fatty acid β-oxidation in *Saccharomyces cerevisiae*. Genes with an oleate response element (ORE box) in their promotor (CGG-N14/N19-CCG) are induced by growth on oleate (+, present; –, absent).

Gene name	ORF	Localization	ORE box CGG-N14/N19-CCG +/–	Enzyme activity
FOX1	YGL205w	peroxisomal matrix	+	acyl-CoA oxidase
FOX2	YKR009c	peroxisomal matrix	+	bi- or multifunctional protein
FOX3	TIL160c	peroxisomal matrix	+	3-ketoacyl-CoA thiolase
SPS19	YNL202w	peroxisomal matrix	+	2,4-dienoyl-CoA reductase
DCI1	YOR180c (EHD2; ECI2)	peroxisomal matrix	+	$\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase
ECI1 (EHD1)	YLR284c	peroxisomal matrix	+	Δ^{3}, Δ^{2}-enoyl-CoA isomerase
FAA1	YOR317w	cytosol	–	acyl-CoA synthetase
FAA2 (FAM1)	YER015w	peroxisomal matrix	+	acyl-CoA synthetase
FAA4	YMR246w	cytosol	–	acyl-CoA synthetase
FAT1	YBR041w	plasma/microsomal and peroxisomal membrane	–	very long chain acyl-CoA synthetase (fatty acid transporter)
FAT2	YBR222c	peroxisomal periphery	+	fatty acid transporter
ACS1	YAL054c	peroxisomal matrix also reported to be mitochondrial	–	acetyl-CoA synthetase
ACB1	YGR037c	cytosol	–	acyl-CoA binding protein
TES1	YJR019c	peroxisomal matrix	+	peroxisomal thioesterase
CAT2	YML042w	peroxisomal matrix	+	carnitine acetyltransferase
CTA1	YDR256c	peroxisomal matrix	+	catalase A
MDH2	YOl126c	cytosol	–	malate dehydrogenase
MDH3	YDL078c	peroxisomal matrix	+	malate dehydrogenase
IDP2	YLR174w	cytosol	–	isocitrate dehydrogenase (NADP)
IDP3	YNL009w	peroxisomal matrix	+	isocitrate dehydrogenase (NADP)
ACO1	YLR304c	cytosol, mitochondrial matrix	–	aconitate hydratase
CIT2	YCR005c	peroxisomal matrix	–	citrate synthase
MLS1	YNL117w	cytosolic and peroxisomal matrix	–	malate synthase
ICL1	YER065c	cytosolic	–	isocitrate lyase
AAT2	YLR027c	cytosolic and peroxisomal	–	aspartate amino transferase
PXA1 (SSH2;Pat2)	YPL147w	peroxisomal membrane	+	ABC transporter
PXA2 (Pat1)	YKL188c	peroxisomal membrane	+	ABC transporter
ANT1	YPR128c	peroxisomal membrane	+	ATP carrier
PEX11/ PMP27	YOL147c	peroxisomal periphery or membrane	+	peroxisomal proliferation and FFA transport pathway
CRC1 (CACT)	YOR100c	mitochondrial inner membrane	+	carnitine/acylcarnitine carrier
SFC1 (ACR1)	YJR095w	mitochondrial inner membrane	+	succinate/fumarate antiporter
AGP2	YBR132c	plasma membrane	+	carnitine carrier

been identified, which catalyzes the dehydrogenation of all acyl-CoAs. During this reaction, hydrogen peroxide is produced, which is subsequently decomposed by catalase to produce H_2O and O_2. ScFox1p is the ortholog of the human acyl-CoA oxidase 1, and its expression is strongly induced by fatty acids [12, 13].

Two different acyl-CoA oxidases have been identified in human peroxisomes [14, 15] and three in rat peroxisomes [16–20]. Due to the possession of different acyl-CoA oxidases, mammalian peroxisomes can oxidize not only straight-chain acyl-CoAs but also 2-methyl branched acyl-CoAs, which is in marked contrast to the situation in *S. cerevisiae*. The ScFox1p accepts long-chain, medium-chain as well as short-chain acyl-CoA substrates, thereby allowing complete degradation of fatty acids to acetyl-CoA units. Mammalian oxidases preferentially react with medium-, long- and very long chain acyl-CoA esters and cannot accept short-chain substrates such as butyryl-CoA (C4) [21].

The second and third step of the peroxisomal β-oxidation spiral

The second and third reactions in β-oxidation are catalyzed by a multifunctional enzyme with both 2-enoyl-CoA hydratase activity and 3-hydroxyacyl-CoA dehydrogenase activity, which results in generation of 3-ketoacyl-CoA from trans-2-enoyl-CoA with 3-hydroxyacyl-CoA as intermediate. ScFox2p (table 1) is a multifunctional enzyme having the activities of 2-enoyl-CoA hydratase-2, (*3R*)-specific 3-hydroxy acyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase, whose latter activity was postulated to convert (*3R*)-hydroxy-CoA to (*3S*)-hydroxyacyl-CoA and vice versa [22].

In human peroxisomes, two different multifunctional proteins with both 2-enoyl-CoA hydratase activity and 3-hydroxyacyl-CoA dehydrogenase activity have been identified that catalyze the conversion of trans-2-enoyl-CoAs to the corresponding 3-keto intermediates either with *L*-3-hydroxyacyl-CoA as intermediate or *D*-3-hydroxyacyl-CoA.

Although ScFox2p and mammalian 2-enoyl-CoA hydratase-2 and (*D*) 3-hydroxyacyl-CoA dehydrogenase (*D*-BP) are related with respect to their amino acid sequences, they possess different kinetic properties. ScFox2p accepts short-chain 2-enoyl-CoA as substrate, thereby allowing β-oxidation to completion, whereas the mammalian multifunctional proteins prefer medium-, long- and very long chain acyl-CoA esters [23, 24].

The final step of the peroxisomal β-oxidation spiral

The final reaction of the peroxisomal β-oxidation pathway in *S. cerevisiae* is catalyzed by a single 3-ketoacyl-CoA thiolase (ScFox3p or ScPot1p) (table 1) [25], which thiolytically cleaves 3-ketoacyl-CoA esters into a C2-shortened acyl-CoA and acetyl-CoA. The expression of thiolase is induced by fatty acids [26]. For humans, two peroxisomal 3-ketoacyl-CoA thiolases are known, including the classical thiolase [27] and a thiolase that is part of the sterol carrier protein SCPx [28]. Both thiolases have overlapping activities.

β-Oxidation of unsaturated fatty acid

S. cerevisiae can utilize both cis- and trans-unsaturated fatty acids as sole carbon source. Saturated and unsaturated fatty acids with a trans double bond at the even-numbered position are direct substrates for the classical β-oxidation spiral. β-Oxidation of unsaturated fatty acids with trans and cis double bonds at odd-numbered positions or cis double bonds at even positions, however, requires the participation of additional enzymes (table 1), including $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (Dci1p), 2,4-dienoyl-CoA reductase (Sps19p) [29] and Δ^3, Δ^2 -enoyl-CoA isomerase (Eci1p) [30]. Figure 2 depicts the degradation of unsaturated fatty acids and the involvement of the various enzymes. Three different routes can be distinguished. Route A is followed by fatty acids with unsaturated bonds at even-numbered positions, yielding 2,4-dienoyl-CoAs (trans-2, cis/trans-4) which can only be further oxidized after conversion to trans-2-enoyl-CoAs by the sequential action of 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase. Fatty acids with unsaturated bonds at odd-numbered positions can follow two different routes. The first route involves conversion of 2,5-dienoyl-CoAs (trans-2, trans/c*is*-5) into trans-2-enoyl-CoAs via the subsequent action of ScFox2p, ScFox3p and Δ^3, Δ^2-enoyl-CoA isomerase. The other NADPH-dependent pathway of 2,5-dienoyl-CoAs (cis/trans-2, cis/trans-5) involves the sequential action of Δ^3, Δ^2,-enoyl-CoA isomerase, $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase, 2,4-dienoyl-CoA reductase and, again, Δ^3, Δ^2, -enoyl-CoA isomerase to finally yield trans-2-enoyl-CoAs.

The degradation of fatty acids with cis double bonds at even positions as in petroselinic acid (C18:1, cis-6) is much slower than the β-oxidation of linoleic acid (C18:2,cis-9,12), although the degradation of both fatty acids depends on 2,4-dienoyl-CoA reductase (Sps19p) [22, 31].

Induction of the peroxisomal fatty acid β-oxidation system

The induction of genes encoding peroxisomal proteins in yeast cells is mediated by the transcription factors Pip2p and Oaf1p, which are involved in particular in the control of expression of genes involved in fatty acid β-oxidation. Although many proteins of the protein import machinery

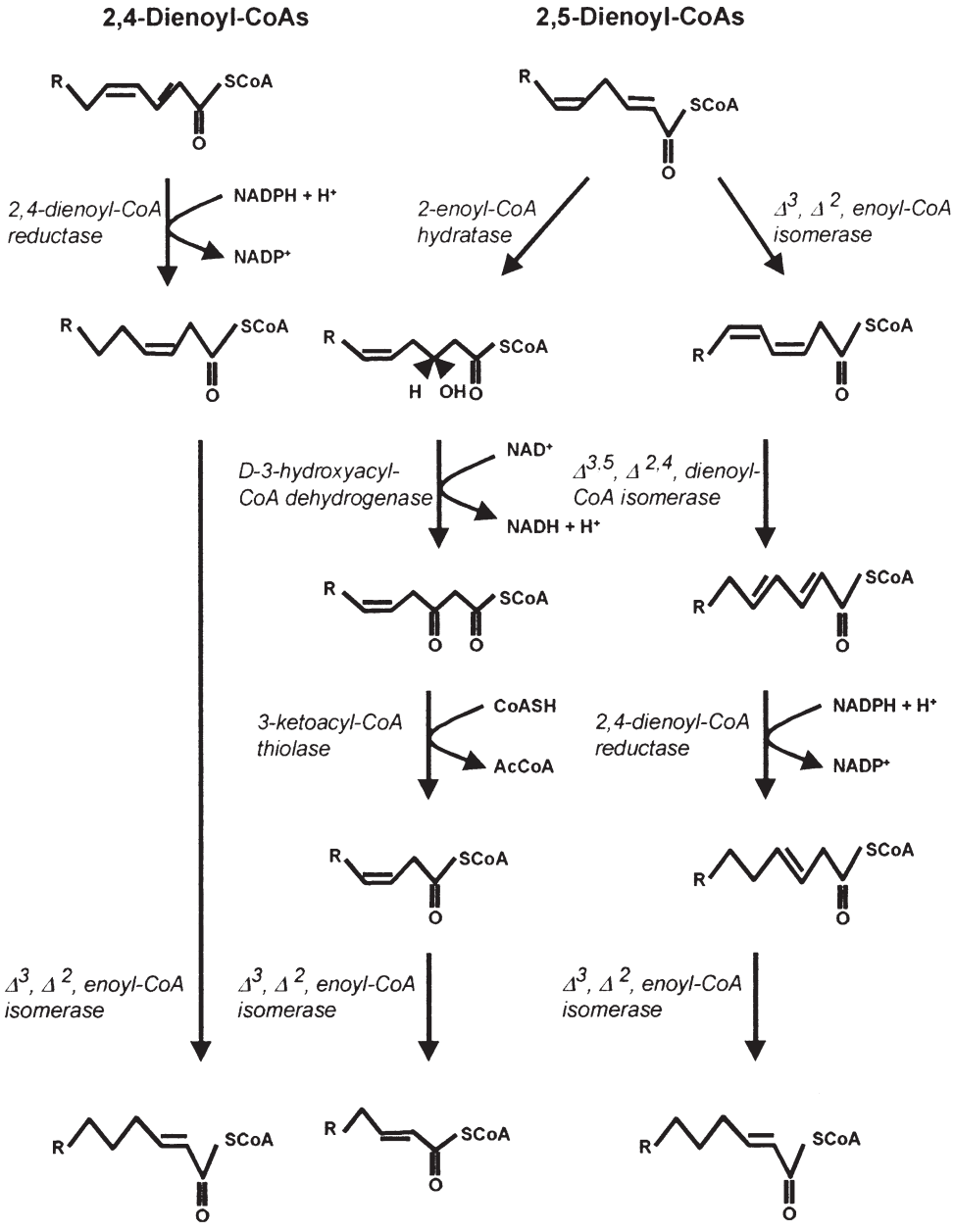

Figure 2. Unsaturated fatty acid oxidation in *S. cerevisiae.* β-Oxidation of unsaturated fatty acids requires the participation of additional enzymes (table 1), including $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (Dci1p), 2,4-dienoyl-CoA reductase (Sps19p) [29] and Δ^3, Δ^2-enoyl-CoA isomerase (Eci1p) [30].

and many of the peroxisomal enzymes are conserved from yeast to humans, the mammalian (PPAR and RXR) and yeast transcription factors (Pip2p and Oaf1p) which control fatty acid oxidation have little in common. The class of nuclear receptors to which peroxisome proliferator-activated receptor α (PPARα) belongs is unknown in yeast, and Zn_2Cys_6 transcription factors are typical of fungi [32]. In *S. cerevisiae*, proliferation of peroxisomes and induction of the fatty acid β-oxidation machinery is required in order to grow on oleate. The promoter sequences of genes coding for yeast proteins involved in fatty acid oxidation contain a positive cis-acting element that mediates the induction of these genes by fatty acids in the medium. This element is called an oleate response element (ORE) [26] (table 1) and consists of an imperfect inverted repeat containing conserved CGG triplets that are spaced by 14–19 nucleotides (CGG-N14/N19-CCG). Further studies demonstrated that Pip2p and Oaf1p interact with each other [33] and form a heterodimer that binds OREs [13] and are required for fatty acid-induced peroxisomal proliferation and regulating the expression of proteins required for fatty acid oxidation.

Overexpression of peroxisomal matrix proteins is not sufficient to induce proliferation of the peroxisomal compartment. This is illustrated by the overexpression of *MDH3* under glucose conditions, which results only in an increased size of existing peroxisomes without inducing peroxisomal proliferation [34]. Therefore, other mechanisms are required to trigger peroxisomal proliferation.

Pex11p has been implicated in the regulation of the number of peroxisomes [35, 36]. Yeast mutants lacking the *PEX11* gene are unable to increase the number of peroxisomes when grown on oleate-containing media and instead accumulate a few giant peroxisomes. On the other hand, cells overexpressing Pex11p exhibit a large number of peroxisomal structures [35–37]. Proteins with amino acid sequence similarity (20%) to *S. cerevisiae* Pex11p have been found in a wide variety of eukaryotes. Overexpression of these homologs in *Kinetoplastida* and mammals also increased peroxisome abundance, which suggests that all these proteins are orthologs [38–40]. It has been suggested that Pex11p is involved in a process leading to fission or vesiculation of preexisting peroxisomes. An observation supporting such a role for Pex11p was made by Passreiter et al. [39], who showed that rat Pex11p can bind coatomer in vitro by virtue of its cytoplasmically exposed carboxyl-terminal dilysine motif. Recruitment of coatomer by Pex11p has been proposed to initiate vesiculation of peroxisomes and as such influence peroxisome proliferation [39]. However, this dilysine motif is not conserved in other Pex11p homologs, including Pex11p from *S. cerevisiae,* thereby raising doubt about the universality of the proposed mechanism for Pex11p-mediated peroxisomal fission. Other motifs might also be present in ScPex11p to recruit proteins for vesiculation. We recently found that Pex11p is required for medium-chain fatty acid oxidation and primarily plays a metabolic role, which affects peroxisome fission [41]. Barnett et al. observed a significant amino acid sequence similarity (30% identity; 50% similarity) between amino acids 2 and 187 of Pex11p and the ligand binding domain (LBD) of the nuclear receptor PPARα, which may suggest that Pex11p binds a similar ligand [42]. Studies in human cells, as well as in the yeasts *Yarrowia lipolytica*, *Candida boidinii* and *S. cerevisiae* support the hypothesis that β-oxidation is required for proper regulation of size and morphology of the peroxisomal compartment. In agreement with this postulate, we found defective peroxisome proliferation in Δ*fox1*, Δ*fox2* and Δ*fox3* cells (data not shown).

Finally, we observed similar giant peroxisomes in an *ant1*Δ mutant defective in ATP transport (see below) growing on oleate. These observations suggest that Ant1p, as well as Pex11p and peroxisomal β-oxidation per se, are involved in the same metabolic pathway that is required for proper peroxisomal fission.

Recent data suggest that *S. cerevisiae* Pex11p is directly involved in peroxisomal proliferation irrespective of the carbon flux through β-oxidation, since overexpression of Pex11p induces peroxisomal proliferation in the absence of fatty acids from the growth medium [43]. It has therefore been suggested that the observed effects of deleting the *PEX11* gene on fatty acid metabolism under basal conditions may be indirect.

In mammals, three Pex11p molecules, Pex11α [44], [39] Pex11β gene and Pex11γ, have been described which are postulated to control peroxisomal proliferation under induced and basal conditions, respectively [40, 44]. Pex11α is required for peroxisomal proliferation in response to 4-phenylbutyrate but is dispensable for PPARα-mediated peroxisomal proliferation [45]. Interestingly, disruption of the *PEX11*β gene is lethal in mice and impairs neural migration but does not abrogate peroxisomal function, which suggests different functions of Pex11α and β [46]. Hoepfner et al. [47] recently demonstrated that in yeast lacking the dynamin-related protein Vps1, the number of peroxisomes was reduced and the peroxisomes appear as large tubular structures. Based on these observations, the authors suggest that Vps1p may be involved in peroxisomal fission and, consequently, in the regulation of peroxisomal abundance in yeast. Koch et al. [48] also reported a dynamine-like protein, DLP1, involved in peroxisome fission in mammals. They demonstrated that peroxisome fission requires a functional Dlp1p, indicating that Pex11βp, which is supposed to control constitutive peroxisome abundance in mammals, is not directly mediating the vesiculation process of the organelle. They propose a direct role for dynamin-like protein Dlp1p in peroxisome fission and in the maintenance of peroxisome morphology in mammalian cells. It is not clear at this moment whether the yeast Vps1p and the mammalian Dlp1p are functional orthologs of each other; however, they share significant amino acid sequence similarity [48].

ScPex11p might be involved in the process of a signaling event that modulates peroxisome proliferation and/or a process to change or modify the peroxisomal membrane composition. Koch et al. [48] speculate that a membrane-modifying activity of Pex11βp could initiate and/or favor the binding of Dlp1p and other factors of the fission machinery to the peroxisomal membrane.

Peroxisomal membrane proteins involved in solute transport

Several peroxisomal membrane proteins (PMPs) have been identified which are presumed to be involved in solute transport across the peroxisomal membrane (table 1). Among them is the *S. cerevisiae* Pmp34p, which is a homolog of the earlier identified PMP47 of *Candida boidinii*. This Pmp47p is a 47-kDa integral protein which shows significant sequence similarity with solute carriers

present in the mitochondrial membrane (MCF) [49]. The N and C termini of Pmp47p were shown to be exposed to the peroxisomal matrix [50], implying an inverted topology when compared with mitochondrial solute carriers [51]. As discussed later, Pmp47p and its orthologs in *S. cerevisiae* and humans are probably involved in transmembrane ATP transport. Another peroxisomal membrane protein with similarity to members of the MCF family has been identified in the rabbit, namely a 53-kDa Ca^{2+}-dependent solute carrier [52]. No homologs of this protein have been found in yeast.

- *PXA1* and *PXA2,* two members of the ATP binding cassette (ABC) transporter family

Two peroxisomal ABC transporters have been identified, namely Pxa1p and Pxa2p [53–55] that are probably involved in the transport of acyl-CoAs (see below). These proteins show high similarity to human PMP70p and ALDp.

- Pmp20p

Finally, one additional peroxisomal membrane protein has been identified in *S. cerevisiae*, i.e. Pmp20p [56, 57]. The function of this PMP is yet unknown.

Transport of metabolites across the peroxisomal membrane

Most of the peroxisomal metabolic pathways require the participation of other cellular components, including the mitochondria, endoplasmic reticulum and/or cytosol. For continuation of β-oxidation, products have to be recycled and/or transported across the peroxisomal membrane. Initially, it had been assumed that peroxisomes are freely permeable to low molecular weight compounds. Although it remains to be resolved why isolated peroxisomes behave as freely permeable organelles, it is now well established that peroxisomes are closed compartments under in vivo conditions. Indeed, we have found that the peroxisomal membrane is impermeable to nicotinamide adenine dinucleotide [NAD(H)] and acetyl-CoA [58] and nicotinamide adenine dinucleotide phosphate [NADP(H)] [31]. These findings predict the existence of metabolite carriers in the peroxisomal membrane to shuttle metabolites from peroxisomes to cytoplasm and visa versa, including substrates for and products from the β-oxidation of fatty acids.

Despite the importance of peroxisomes and the substantial knowledge of their biochemical properties, little is known about the ionic composition of the peroxisomal lumen. Using P-NMR (nuclear magnetic resonance), Nicolay et al. [59] observed an acidic peroxisomal lumen in different methylotrophic yeast strains. Moreover, Douma et al. [60] reported the existence of a putative proton-translocating ATPase in the peroxisomal membrane of one of these yeasts, *Hansenula polymorpha*, that may be responsible for generating the pH gradient.

The results obtained in yeast are in contrast with recent publications of Dansen et al. [61] and Jankowski et al. [62] in mammalian cells, and Van der Lende et al. [63] in *Penicillium chrysogenum*, who reported that the intraperoxisomal pH is either basic or resembling the cytosolic pH, respectively. Importantly, we also observed a basic pH in peroxisomes of *S. cerevisiae* when cultured on oleate [C. W. T. van Roermund et al., unpublished].

Fatty acid transport

Uptake and activation

Transport of unesterified free fatty acids (FFAs) has been proposed to proceed via simple diffusion mainly regulated by the rules of lipid physical chemistry. Recently, however, a more complex process involving protein catalysis has been suggested. Hamilton [64] divided FFA transport in cell membranes into three essential steps: adsorption, transmembrane movement and desorption. Because of their low solubility in water and high hydrophobicity, fatty acids bind rapidly and avidly to model membranes: if albumin is a donor, FFAs desorb rapidly to reach their equilibrium distribution. Desorption of FFA from a phospholipid surface is slower than transmembrane movement and is dependent on the FFA chain length and unsaturation, but rapid for typical dietary FFA. The physical properties of FFA in model systems predict that proteins are not essential for transport of FFA through membranes. Various factors can influence the rate of passive fatty acid movement across a membrane: (i) a transmembrane pH gradient [65], (ii) the relative distribution of fatty acid binding sites on both sides of the membrane [66], (iii) modification of free fatty acids to membrane impermeable derivatives (acyl-CoA esters) on the trans side of the membrane [67, 68] and (iv) utilization of fatty acids for anabolic and catabolic processes, thereby creating a sink.

Fatty acids can cross the membrane either by virtue of their solubility or by being actively taken up by cells in a process mediated by proteins of the fatty acid transport (FATP) family. The protein encoded by the yeast *FAT1* gene has 54% overall similarity to human FATP and contains an adenosine monophosphate (AMP) binding motif common to such proteins as acyl-CoA synthetases. When the fatty acid synthase inhibitor cerulenin is added to the medium, yeast cells stop growing but can be rescued by supplementing different fatty acids. Cells carrying a disruption of the FAT1 gene (Δ*fat1*), however, have difficulty growing in the presence of cerulenin even in the presence of fatty acids. Incorporation of fatty acids into lipids was also impaired in the Δ*fat1* cells. Thus, based on these data, Fat1p was proposed to function as a fatty acid transporter protein similar to that proposed for the murine FATP [68, 69]. Recent observations, however, indicate

that the impaired fatty acid uptake observed in the Δ*fat1* cells is secondary to a defect in the metabolism of the fatty acid. Subcellular experiments suggest that ScFat1p is associated with the endoplasmic reticulum and peroxisomal membrane, rather than the plasma membrane [70, 71]. Furthermore, heterologous expression of the *FAT1* gene indicates that Fat1p is a very long chain acyl-CoA synthetase (VLCS) [70, 71]. ScFat1p appears to be involved in the maintenance of very long chain fatty acid homeostasis, only indirectly affecting utilization of exogenous fatty acids. It is interesting that murine FATP has also recently been reported to be a VLCS [72] and can complement the Fat1-deficient phenotype [73]. A subsequent study based on site-directed mutagenesis demonstrated that VLCFA-synthetase activity of Fat1p could be separated from its function in fatty acid transport. This result appears to indicate that Fat1p also plays a direct role in fatty acid transport [74]. Five additional genes (*FAA1–4* and *FAT2*) encoding proteins with similarity to acyl-CoA synthetases have been described in *S. cerevisiae* [70, 75]. The *FAA1* and *FAA4* genes encode acyl-CoA synthetases (table 1) required for activation of imported exogenous fatty acids [76–78]. Faa1p and Faa4p account for 99% of total 14:0 and 16:0 activation activity in *S. cerevisiae,* and when endogenous fatty acid synthesis is blocked, at least one is required for rescue on medium containing exogenous fatty acids [78]. Cells carrying disruptions in both genes (Δ*faa1/ faa4*) appear to have normal initial rates of free fatty acid import [78], but bulk accumulation in cell lipids is negligible [71]. Thus, it seems that these cells are defective in the activation, but not the transport, of the fatty acids. However, Choi and Martin [71] attempted to repeat the experiments of Knoll et al. [78] by specifically inactivating fatty acid synthetase through gene disruption. In the case of the Δ*faa1/ faa4* strain, growth inhibition appears to be caused by a simple failure to import sufficient oleate to sustain growth. This suggests the involvement of both genes in fatty acid metabolism, since the double knockout (Δ*faa1/ faa4*) fails to grow on oleate as sole carbon source.

Disruption of the *FAA3* and *FAA2* genes has no affect on the ability of cells to use exogenously supplied fatty acids, which suggests that the acyl-CoA synthetases encoded by these genes can access only fatty acids synthesized within the cell [77]. Faa2p has been localized to the matrix side of the peroxisomal membrane [55] and accounts for the residual VLCS activity present in cells lacking Fat1p [71].

The *FAT2* gene product (Fat2p, previously named Psc60p) is located in the peroxisomal matrix and associated with the peroxisomal membrane but is not required for growth on oleic acid [75]. The cellular roles of Faa3p and Fat2p are not clear.

In the cytoplasm, acyl-CoA esters and free fatty acids are bound to binding proteins named ACBPs and FABPs, respectively. Besides protecting the cellular membranes from free fatty acids, FABPs and ACBPs have been postulated to play a role in the delivery of fatty acids and acyl-CoA esters to various compartments and fatty acid-consuming systems [69, 79, 80].

The yeast *S. cerevisiae* only possesses one *ACBP* gene, which encodes a protein called Acb1p [81]. It is still a matter of debate whether Acb1p is involved in the transport of acyl-CoA to the peroxisomes for oxidation or to the cytosol for fatty acid elongation [82, 83].

Fatty acid transport across the peroxisomal membrane

The substrates for β-oxidation can enter the peroxisome via two different pathways (fig. 1) [55]. First, fatty acids such as MCFAs enter peroxisomes as free fatty acids and are subsequently activated by the peroxisomal acyl-CoA synthetase, Faa2p. Pex11p is also required for β-oxidation of fatty acids that enter peroxisomes as free fatty acids [84]. As discussed above, Pex11p shows extensive amino acid sequence similarity to the LBD of the nuclear hormone receptor [42], suggesting that Pex11p might contain a binding site for fatty acids, which perhaps participates in fatty acid transport across the peroxisomal membrane.

The second way of fatty acids to enter the peroxisome is as activated CoA esters. This activation occurs outside peroxisomes catalyzed by extraperoxisomal synthetases followed by uptake of the CoA esters via Pxa1/Pxa2p [53–55]. This pathway is used predominantly for import of LCFAs like oleate [55].

Pxa1p and Pxa2p are peroxisomal membrane proteins that comprise the two halves of an ABC transporter required for the peroxisomal transport of activated long-chain fatty acids. It has been hypothesized that Pxa1p/Pxa2p functions as an acyl-CoA flippase in the peroxisomal membrane [53, 85]. Membrane-inserted acyl-CoA esters could then diffuse in the plane of the bilayer until they bind Pxa1p/Pxa2p, which flip the polar CoA group from the cytoplasmic leaflet to the luminal leaflet of the peroxisomal membrane in an ATP-dependent manner [86].

A number of different candidate transport proteins with similarity to the yeast Pxa1p and Pxa2p have been identified in mammalian peroxisomes. The first one was identified by Kamijo et al. [87] and involves a 70-kDa peroxisomal membrane protein, a member of the superfamily of ABC proteins. Studies by Mosser et al. [88] led to the identification of a second peroxisomal membrane protein belonging to the ABC superfamily, the adrenoleukodystrophy (ALD) protein (ALDp). In human cells, two additional half-size transporters with marked homology to ALDp and PMP70p have recently been identified [88–90]: ALD-related protein (ALDRp) and 69-kDa pro-

tein (PMP69p), respectively. The functions of the peroxisomal ABC half-transporters and their interaction with VLCFA synthetase is unknown, but their considerable sequence similarity indicates that they might have related and/or overlapping functions in peroxisomal fatty acid metabolism. It has been suggested that functional transporters heterodimerize from different sets of half-size transporters to provide distinct functions in different tissues [91].

Shuttling of metabolites across the peroxisomal membrane

Reoxidation of intraperoxisomal NADH

Previously, we provided evidence for a major role of peroxisomal malate dehydrogenase (Mdh3p) in intraperoxisomal NAD^+ regeneration. This was concluded from the observation that deletion of the *MDH3* gene, encoding peroxisomal malate dehydrogenase, leads to deficient fatty acid oxidation in intact cells. Furthermore, there was accumulation of 3-hydroxyacyl-CoA esters. These results indicate that Δ*mdh3* cells are blocked at the NAD^+-dependent dehydrogenation step. The Mdh3p-dependent regeneration of NAD^+ depends on the presence of oxaloacetate inside peroxisomes. It has been hypothesized that the reducing equivalents are shuttled via aspartate/malate, similar to the situation in mitochondria (fig. 3) [92]. The additional enzyme activity required for this shuttle, i.e. aspartate aminotransferase, was found to be localized inside peroxisomes, at least partially, under oleate conditions [34]. However, aspartate aminotransferase is not involved in NADH-oxidation per se, since disruption of the corresponding gene (*AAT2*) did not affect fatty acid β-oxidation [34]. The finding that Δ*mdh3* cells are not impaired in growth on acetate suggests that Mdh3p does not participate in glyoxylate cycle activity. Indications that the kinetic parameters of glyoxysomal malate dehydrogenase are unfavorable to its participation in the glyoxylate cycle of plant glyoxysomes were earlier reported by Mettler and Beevers [93]. The consequence of these findings is that malate produced by the glyoxylate cycle is transported out of the peroxisome followed by retroconversion to oxaloacetate in the cytosol (via Mdh2p) or mitochondria (via Mdh1p).

Reduction of intraperoxisomal $NADP^+$

β-Oxidation of some unsaturated fatty acids requires the participation of the enzyme 2,4-dienoyl-CoA reductase, which converts 2,4-dienoyl-CoA into Δ*3*-enoyl-CoA with the concomitant production of $NADP^+$ from NADPH. As described by van Roermund et al. [31] and Henke et al. [94] peroxisomal NADP-linked isocitrate dehydrogenase activity plays a key role in the provision of NADPH required for the dienoyl-CoA reductase activity. Disruption of the *IDP3* gene blocked the β-oxidation of unsaturated fatty acids containing an even-numbered double bond (fig. 4). Under these conditions there was accumulation of 2,4-dienoyl-CoAs, indicating that the metabolism of these fatty

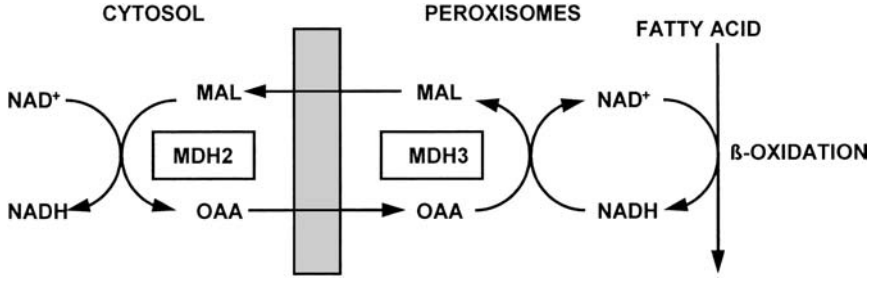

Figure 3. Schematic representation of the proposed metabolic pathway for reoxidation of intraperoxisomal NADH in *S. cerevisiae*. The malate (MAL)-oxaloacetate (OAA) shuttle, exchanging peroxisomal NADH for cytosolic NAD^+.

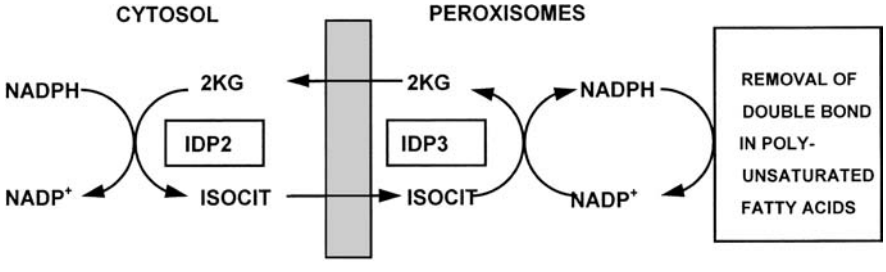

Figure 4. Schematic representation of the proposed metabolic pathway for reduction of intraperoxisomal $NADP^+$. The 2-ketoglutarate (2KG)/isocitrate (ISOCI) shuttle, exchanging peroxisomal $NADP^+$ for cytosolic NADPH.

acids was blocked at the level of the dienoyl-CoA reductase step. β-Oxidation of unsaturated fatty acids with an odd-numbered double bond (like oleate), however, was unaffected, implying that these unsaturated fatty acids can be degraded independent of NADPH-dependent reduction of 5-enoyl-CoA, supporting a role for the isomerase- or diisomerase-dependent pathway in vivo (fig. 2).

Export of acetyl-CoA from the peroxisomal interior to the mitochondrial matrix

Peroxisomal citrate synthase activity (Cit2p) can be eliminated without impairment of growth on acetate or oleate. This is rather surprising considering the essential role of the glyoxylate cycle discussed before. It has been argued that citrate produced in mitochondria under certain conditions can reach the peroxisomes for further metabolism. Alternatively, the assimilation of acetyl-CoA can take place in a somewhat modified form, which bypasses the need for Cit2p. The lack of an oleate-non-utilizer (ONU) phenotype in the Δ*cit2* cells could be explained by the presence of an alternative pathway for export of the acetyl-CoA units produced during β-oxidation, formed by the carnitine acetyltransferase protein (Cat2p). Disruption of both the *CIT2* and *CAT2* genes indeed blocked the β-oxidation of oleate in *S. cerevisiae*. These results led us to postulate two different routes for transport of β-oxidation products from peroxisomes to mitochondria [58]; one via the glyoxylate cycle, and another via the carnitine transport pathway (fig. 5).

Using a selective screen, we have isolated several mutants that are specifically affected in the second pathway, the carnitine-dependent acetyl-CoA transport (CDAT) from the peroxisomes to the mitochondria, and assigned these CDAT mutants to three different complementation groups. The corresponding genes were identified using functional complementation of the mutants with a genomic DNA library. In addition to the previously reported carnitine acetyl-CoA transferase gene (*CAT2*), we identified the genes for the yeast ortholog of the human mitochondrial carnitine acylcarnitine translocase (*CRC1*) and for a transport protein (*AGP2*) required for carnitine transport across the plasma membrane. Although these studies predicted the existence of a similar carrier involved in the export of acetylcarnitine in the peroxisomal membrane, so far no candidate genes have been identified. Frazer and Zammit [95] have suggested that Crc1p has a dual subcellular localization both in mitochondria and peroxisomes. However, making use of specific antibodies raised against Crc1p, we have found no evidence for a dual localization of Crc1p [C. W. T. van Roermund, unpublished].

As mentioned previously, fatty acids are degraded to completion in yeast peroxisomes, without involvement of mitochondria. The situation in mammals is different, due to the incomplete oxidation of fatty acids in peroxisomes. It has been demonstrated that at least some chain-shortened acyl-CoAs are transported to mitochondria in the form of a carnitine ester [96], which involves the participation of the peroxisomal carnitine octanoyl-CoA transferase activity (Cotp) [97, 98]. Alternatively, hydrolysis of acyl-CoAs within peroxisomes is catalyzed either by acyl-CoA thioesterase or hydrolases [99, 100]. An additional role of thioesterase suggested by Hunt et al. [101,

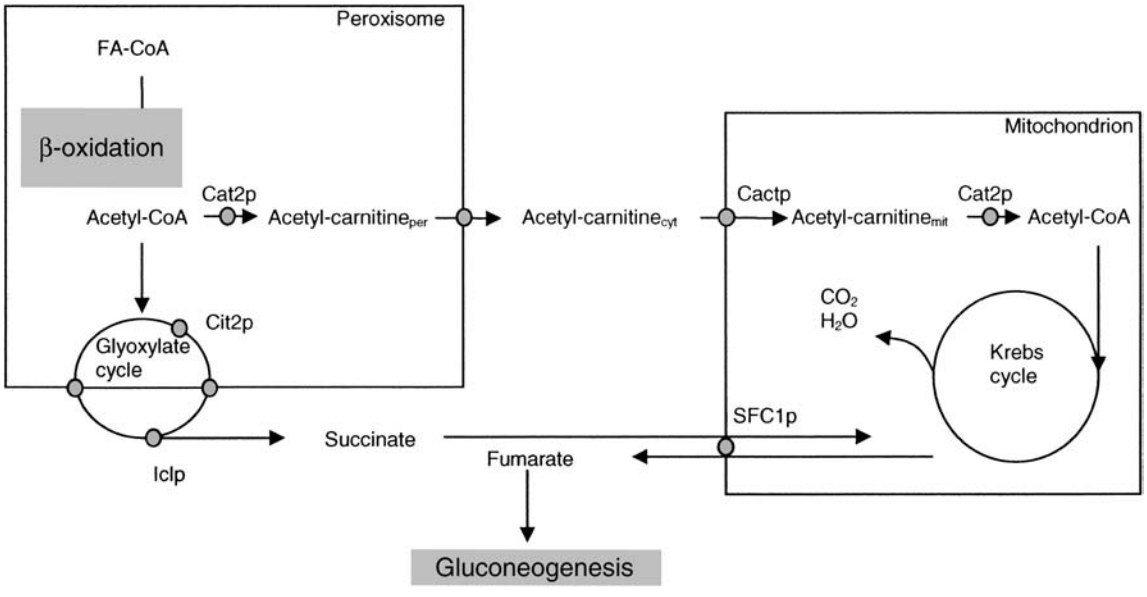

Figure 5. Export of acetyl-CoA from peroxisomes to mitochondria during fatty acid oxidation in *S. cerevisiae* (see table 1 for abbreviations).

102] is the regulation of the CoA levels inside the various thioesterase-containing organelles. By controlling the CoA levels, thioesterase could regulate the β-oxidation activity inside mitochondria or peroxisomes.
S. cerevisiae peroxisomes contain at least one acyl-CoA thioesterase, named Pte1p/Tesp. Its role in β-oxidation is currently not well understood, particularly since there is ambiguity in literature about the phenotype of the Δ*tes* mutant. Indeed, according to Kal et al. [103] the Δ*tes* mutant does not show impaired growth on oleate, in contrast to the results of Jones et al. [104].

ATP transport across the peroxisomal membrane

The yeast *S. cerevisiae* genome contains 35 genes encoding proteins belonging to the mitochondrial carrier family (MCF). At least one of these MCFs (Ant1p) is located in peroxisomes [50, 105]. Disruption of the *ANT1* gene results in impaired growth of the yeast when the medium-chain fatty acid (MCFA) laureate was used as a single carbon source, whereas normal growth was observed with the long-chain fatty acid (LCFA) oleate [84, 106]. These results imply that a transport step specific for MCFA β-oxidation is impaired in *ant1*Δ cells. Since MCFA β-oxidation in peroxisomes requires both ATP and CoASH for activation of the MCFAs into their corresponding CoA esters, different investigators studied whether Ant1p is an ATP carrier (fig. 1) [50, 106]. We used firefly luciferase targeted to peroxisomes to measure ATP consumption inside peroxisomes and showed that peroxisomal luciferase activity was strongly reduced in intact *ant1*Δ mutant cells when compared with wild-type cells but comparable in lysates of both cell strains. This led us to conclude that Ant1p most likely mediates the transport of ATP across the peroxisomal membrane. Recently, direct evidence for the role of Ant1p as ATP transporter was provided by Palmieri et al. [107], who functionally reconstituted the purified carrier into liposomes. Ant1p was able to exchange ATP for ADP, but also AMP, which clearly differs from the substrate specificity of the mitochondrial ADP/ATP exchanger [108].
Wylin et al. [109] and Jones et al. [110] demonstrated that the human homolog of Ant1p was located in peroxisomes. Recent studies by Visser et al. [111] showed that the human Ant1p is also an adenine nucleotide transporter. This is concluded from different experimental findings, including rescue of the defect in MCFA oxidation in *S. cerevisiae* cells in which *ANT1* was disrupted. Direct proof was provided when purified Ant1p was reconstituted in liposomes, followed by the demonstration of ATP uptake.

Concluding remarks

In recent years much has been learned about the fatty acid oxidation system in yeast, including the transport of fatty acids and other metabolites required for β-oxidation across the peroxisomal membrane. Much remains to be learned, however, especially about the exact way various metabolites are transported across the membrane.
One goal of future research will be to understand how these transport steps shuttle metabolites across the peroxisomal membrane and which genes are involved. In fact, many peroxisomal transporters remain to be identified that are involved in the efficient communication between peroxisomes and other cellular compartments.

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